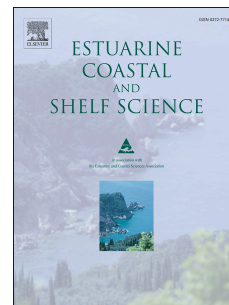


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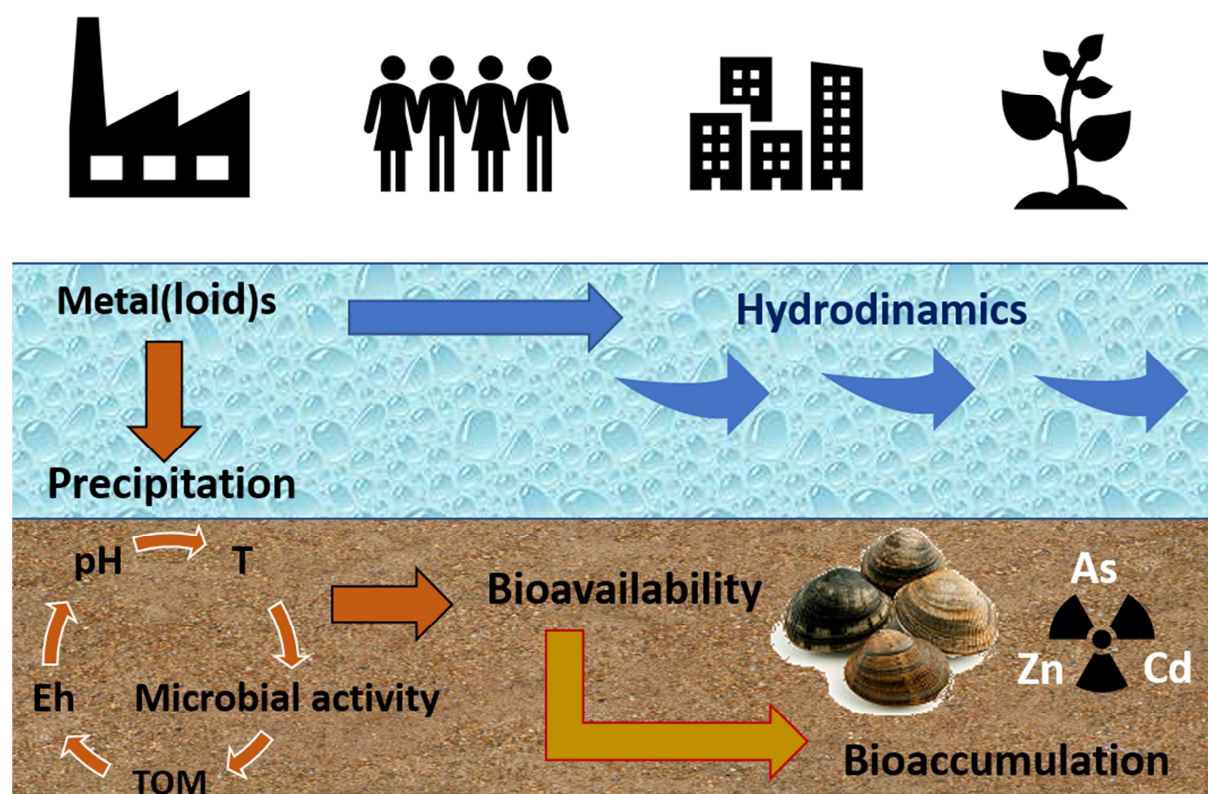
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**Biogeochemical dynamics and bioaccumulation processes in Manila clam: implications for biodiversity and ecosystem services in the Ria de Aveiro Lagoon**

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**Keywords:** Geochemical speciation, metal(loid) content, bioaccumulation, *Ruditapes philippinarum*, Ria de Aveiro Lagoon.

**Abstract**

The present work was carried out in the Ria de Aveiro Lagoon (Portugal) to better understand the dynamics driving the bioaccumulation processes in edible bivalves, namely in the Manila clam *Ruditapes philippinarum*. For the first time, a holistic approach was applied, collecting data on sediment physico-chemical characteristics and its contamination, geochemical speciation and metal(loid) bioaccumulation in clams, from three exploited areas of the lagoon (Costa Nova, Torreira, Ílhavo). The Ria de Aveiro Lagoon is part of the Natura 2000 network, has the designation of Special Protection Area (SPA), contains Sites of Community Importance (SCI), it is protected by the EU Birds Directive (79/109/CEE) and includes a natural reserve in its northern part. Specifically concerning the metal(loid) occurrence, the monitoring and the identification of contaminated sites in protected areas are priorities, to improve the biodiversity conservation efforts and to ensure the correct management of natural resources.

Results showed that multiple driving forces interacted together to determine site- specific impacts, resulting in different risks at local scale for the transferring of the metal(loid)s to the trophic chain. Hydrodynamics played a major role driving the occurrence of depositional or ablative sites, influencing the granulometric composition of sediments and their contamination; metal(loid) chemical forms were then determined by multiple factors like pH, redox potential and organic matter content. The geochemical speciation data showed that the Ílhavo area should be considered the most dangerous in terms of clam consumption, since in case of environmental changes, possible conversion of the elements from the potentially bioavailable forms to the bioavailable ones can occur. Moreover, bioaccumulation processes in Manila clam were observed for Cd, Zn and especially As, the latter representing serious risk for consumer safety throughout clam consumption.

## 1. Introduction

Coastal lagoons are considered as highly unpredictable environments, subjected to spatial and temporal bio-geochemical variability (Ferrarin et al., 2010). Moreover, they are among the most productive ecosystems (Covelli, 2012), deeply exploited for human activities. Anthropogenic pressures like industries, agriculture, sewage discharges and aquaculture has often altered the fresh and saltwater inputs, nutrient loading, sedimentation and accumulation of contaminants (Covelli, 2012). Transitional environments like coastal lagoons are of primarily importance, due to their filter role between the landscape and the Sea, influencing the fate of contaminants in marine ecosystems (Du Laing, 2011). In fact, due to their key role coastal lagoons are also considered as a Priority Habitat for conservation in Europe.

The Ria de Aveiro Lagoon, located in the North-Western Portugal, represents an interesting example of a shallow-water ecosystem. It is formed by several branches and it is characterized by a network of saltmarshes and channels resulting in a very irregular and complex geometry (Martins et al., 2015 a, b). The lagoon has been subjected to anthropogenic pressures (Martins et al., 2015 a,b), particularly a mercury enriched effluent from a chloralkali industry which has contaminated a specific area of this ecosystem, the so called Laranjo Bay (Castro et al., 2009; Freitas et al., 2012 a,b; Pereira et al., 2009). Moreover, as the lagoon has very complex hydrodynamics (Dias et al., 1999) affecting the biological, chemical and geological processes, recent mineralogical and geochemical data collected from this ecosystem showed the occurrence of both low and moderately polluted sites (Martins et al., 2015 a,b; Velez et al., 2015a).

It must be underlined that, particularly in multi-use protected areas, like the Ria de Aveiro Lagoon, it is fundamental to identify the risks related to pollution and transferring of the contaminants among abiotic and biotic compounds, both for biodiversity conservation and human health.

Among contaminants, inorganic compounds such as metals and metalloids are considered particularly dangerous, due to their persistence, toxicity and bioaccumulative capabilities (Du Laing, 2011). Elements are transported by freshwaters as soluble phases or adsorbed on suspended particles (particularly bounded to fine particles and organic matter which are excellent metal(loid)-scavengers) (de Groot et al., 1976; Förstner and Whittmann, 1979; Luoma and Rainbow, 2008). Once they reach the interface between freshwater and saltwater occurring in transitional environments, they are subjected to chemical-physical transformations which may shift their aggregative properties while entering in a different hydric circulation pattern (Du Laing, 2011). Additionally, site-specific hydrological and hydrodynamic conditions may lead to the establishment of specific areas enriched of contaminants, posing risks for the transitional ecosystems. According to the granulometric characteristics, the particles reaching the transitional environments have a different fate: the largest particles keep their individual dimensions and their depositional behavior depends solely on hydrodynamic and gravitational factors (Aston and Chester, 1976). On the opposite, particles of smaller dimensions are influenced by a series of processes (such as coagulation and flocculation), which modify their dimensional distribution and depositional route (Aston and Chester, 1976).

Another important factor to be considered is the occurrence of organic matter which, along with oxides/hydroxides of Fe and Mn, can form flocs with fine-grained particles. Flocs sediment in a colloidal form together with the newly formed aggregates, also drag soluble metal(loid) chemical forms (Argese et al., 1992) and stabilize the majority of them in the sediments (Aston and Chester, 1976; Burton, 1976). Besides granulometry and organic matter, also hydrodynamics, biogeochemical processes and environmental conditions (microbial activities, redox potential, pH, dissolved O<sub>2</sub>, salinity and temperature) can determine both spatial distribution and bioavailability of elements in transitional environments (Gambrell et al., 1980).

Metal(loid)s are distributed among a variety of association forms, differing in the intensity of metal-matrix bonds: as a consequence, each of these forms exhibits a different potential for remobilization

and a different bioavailability (Argese and Bettiol, 2001). It must be stated that metal(loid)s transferring from the soluble phase to the sediment, and from the sediment to the soluble phase, it is not a definitive process, instead it is always changing due to the variable environmental conditions; elements can be continuously solubilized passing from a chemical form to another (Gambrell et al., 1980).

As a consequence, total metal(loid) content data do not provide sufficient information about the real risk for bioaccumulation in biotic matrixes, posed by contaminated sediments. On the opposite, metal(loid) partitioning by geochemical speciation can help in evaluating the amount of directly and/or potentially bioavailable element fractions (Argese et al., 2003).

Edible bivalves are well-know bioindicators which can supply the direct estimation of bioavailability of toxic compounds as metal(loid)s (Argese et al., 2005; Bettiol et al., 2008). They have been widely investigated to determine the risks associated with their consumption (Sfriso et al., 2008; Figueira et al., 2011, 2013; Freitas et al., 2012a,b; Velez et al., 2014; 2015a,b; Chiesa et al., 2018). Among commercially exploited bivalves, Manila clam *Ruditapes philippinarum* - synonym *Venerupis philippinarum* (Adams and Reeve, 1850) plays a major role, exceeding 250,000 tons of production per year (FAO, 2013). Originally distributed in the Indo-Pacific region, it has been introduced in many countries for aquaculture and fisheries (Gosling, 2003), including European Atlantic and Mediterranean coastal waters (Chiesa et al., 2017). In Portugal, the first report dates back to 1984 in Ria Formosa (Algarve), but nowadays it has been reported in many estuarine systems all over the country, including the Tagus and Sado estuaries, the Ria de Aveiro and Óbidos lagoons (Gaspar, 2010; Chainho, 2014; Chainho et al., 2015; Velez et al. 2015a; b).

In order to study the complex equilibrium of metal(loid)s in a transitional environment, a holistic approach on a local scale is required for evaluating the element partitioning, dynamics and accumulation in abiotic and biotic matrixes. This approach would be particularly useful to understand the dynamics regulating the bioaccumulation patterns in edible bivalves, specifically Manila clams, exploited from the Ria de Aveiro Lagoon.

Thus, the aims of the present work are multiple: (i) to determine the total metal(loid) content and their partitioning in the estuarine sediments where Manila clam is occurring; (ii) to determine the metal(loid) bioaccumulation in clams; (iii) to identify the driving forces in their biogeochemical dynamics and (iv) their interactions at local scale.



## 2. Material and methods

### 2.1. Field sampling

#### 2.1.1. Study area

The Ria de Aveiro Lagoon is located in NW Portugal (40°38' N, 8°45' W). It is 45 km long and 10 km wide and covers an area of 83 km<sup>2</sup> at high tide (spring tide), reduced to 66 km<sup>2</sup> at low tide (Figueira et al., 2011). It is characterized by narrow channels and significant intertidal zones, namely mud flats and salt marshes (Dias et al., 2000). Several channels (S. Jacinto, Ílhavo, Mira, Ovar, and Murtosa) are occurring (Velez et al., 2014).

The Ria de Aveiro comprehends a wide range of biotopes (e.g. wetlands, salt marshes and mudflats) used as nursery areas for many valuable species, namely bivalves, crustaceans, fish and birds. Moreover, from the conservational point of view this system is considered a high priority since it is a fundamental step in the migration of aquatic birds and an ideal place for winter shelter and nesting (Reis, 1993).

Ria de Aveiro is part of the Natura 2000 network (EU Habitats Directive), has the designation of Special Protection Area (SPA), with several areas classified as Sites of Community Importance (SCI), and it is protected by the EU Birds Directive (79/109/CEE) (Lillebö et al., 2015). Moreover, in its northern part, between S. Jacinto and Torreira, there is a nature reserve called 'Reserva Natural das Dunas de S. Jacinto' (Lillebö et al., 2015).

It is a multi-estuarine system which receives salt water from Atlantic Ocean through an artificial narrow channel (1.3 Km long, 350 m wide and 20 m deep) and freshwater from several streams and rivers (Dias et al., 2000; Martins et al., 2015a) of different and changing catchment area (from 30 to 300 km<sup>2</sup>), depending on precipitation frequency and intensity (Silva and Duck 2001). The mean tidal range is of about 2.0 m with a minimum of 0.6 (neap tides) and a maximum of 3.2 m (spring tides) (Dias et al., 1999). The average depth of the lagoon is 1 m, except for navigation channels, which are generally 7 m deep (Dias et al., 2000). The tidal prism in each one of the main channels (relative to its value at the mouth) is about 38% for S. Jacinto channel, 26% for Espinheiro channel,

10% for Mira channel and 8% for Ilhavo channel (Silva, 1994). The tide at the mouth is semidiurnal and generates tidal flows strongly influenced by local geometry. Tidal distortions have been observed at the extremities of the S. Jacinto and Mira channels as the wave amplitude decrease leading to an increase of the phase lag, so the tidal propagation have the characteristics of a damped progressive wave (Dias et al., 1999).

Thus, the lagoon includes areas nearby the saltwater entrance with stronger hydrodynamic conditions (Martins et al., 2015a,b) where erosion prevails due to intense currents affecting bottom sediments, and shallow and tidal flat zones where fine-grained sediments are deposited and accumulated (Lopes and Dias, 2007).

Martins et al. (2015a) stated that the rivers are the main sources of metal(loid)s introduced into Ria de Aveiro Lagoon, thus the confined areas located in the vicinity of the river mouths constitute hotspots of contamination. Moreover, the northern area of the Ovar Channel and the inner zone of Murtosa Channel have the contribution of several rivers and streams crossing industrialized areas, the major represented by the industrial complex of Estarreja (Martins et al., 2015a). The latter was a chloralkali plant, which introduced a huge amount of pollutants in the Aveiro Lagoon. Despite the emissions ceased in 2004, sediments remain contaminated by high levels of metal(loid)s (Lopes et al., 2014). Through the inner zone of the Murtosa Channel contaminants can be then remobilized, and exported to other areas by currents, adsorbed in suspended particles, such as clay minerals and organic compounds (Martins et al., 2015a).

### **2.1.2. Sampling procedures**

Three sampling areas - Costa Nova, Ílhavo and Torreira - have been selected in the Ria de Aveiro Lagoon (Figure 1), considering previous studies conducted on both *R. philippinarum* distribution and metal(loid) contamination (Velez et al., 2014; 2015a). Moreover, areas have been selected due to the different hydrodynamic patterns, tidal influence and anthropogenic pressures.

Three sites (labelled as A, B, C) were sampled from each investigated area (3x3 = 9 sampling sites in total). Details on sampling locations with GPS coordinates are reported in Table 1.

As for the nine sediment samples, each of them was obtained from the homogenization on the boat of three captures, taken nearby each other while boat was turned off, to avoid hydrocarbons contamination and sediments resuspension.

At each of the nine sampling sites the salinity, pH, T and redox potential (Eh) of the sediments were measured with specific probes.

Professional scuba divers collected one pool of clams from each area -Torreira, Costa Nova and Ílhavo- due to low clam density occurring at the time of the sampling, and the high tides occurring during the campaign.

After sampling, specimens and sediments were transported on ice (0 °C) to the laboratory. Sediments were stored at -20°C until processing, while organisms were preserved at -80 °C until analysis.

## **2.2. Laboratory analyses**

### **2.2.1. Sediment grain size distribution and organic matter content**

Sediment grain size was analyzed by dry sieving without any pre-treatment. This specific procedure was selected to preserve as much as possible the original granulometric distribution, preventing the disruption of aggregates (Zonta et al., 1993) and the loss of organic matter, for subsequent geochemical speciation analyses.

Each sediment sample was freeze-dried and passed through a 2 mm sieve to remove shell or sticks. The silt and clay fraction (fine particles) was obtained by dry sieving through a 63 µm sieve and expressed as a whole, in terms of percentage of the total sediment (dry weight). Then, sediments were dry-sieved through a 25 µm sieve.

Total organic matter content (TOM) was determined according to Byers et al. (1978), as corresponding to the percentage of weight loss in 1 g of dried sediment, after combustion at 450 °C during 5 h (Velez et al., 2015b).

### **2.2.2. Total metal(loid) content in the whole sediments and in the granulometric fractions**

The total concentration of Arsenic (As), Cadmium (Cd), Chromium (Cr), Copper (Cu), Iron (Fe), Manganese (Mn), Nickel (Ni), Zinc (Zn), Lead (Pb) and Mercury (Hg) were measured in each of the nine sediment samples.

The contamination level was investigated both in the whole sediment and in each granulometric fraction, namely  $> 63 \mu\text{m}$ , between 63 and  $25 \mu\text{m}$ , and in the fraction  $< 25 \mu\text{m}$ .

For both whole sediment and granulometric fractions, samples were lyophilized and the determination of total metal(loid)s were performed using the total digestion method (Bettiol et al., 2008): aliquots of about 0.1g of lyophilized sediment samples were microwave-digested in closed Teflon vessels in a CEM MDS-2000 microwave oven, with 5 ml of Milli-Q water, 3 ml of “total digestion solution” composed of aqua regia (1:3  $\text{HNO}_3\text{:HCl}$ ) plus 1.5 ml of HF solution (48%). Element concentrations in the extracts were determined by means of either flame or flameless atomic absorption spectrometry. All analyses were performed in triplicate to assess the analytical reliability, ensuring a S.D. lower than 10%.

Regarding the quality controls, the calibration of the apparatus was made with IV standard solutions and they were verified with reference material (PACS-2 marine sediment – National Research Council Canada).

The concentration of elements in sediments was expressed in mg per kg of dry weight (d.w.), to allow the comparison with sediment quality guideline values (Field et al., 2002).

### **2.2.3. Geochemical speciation**

The geochemical speciation was carried out for the whole sediment. Considering that geochemical speciation is money and time consuming, it was performed on a subset of sediment samples, representing different granulometric composition, Eh values and total element contamination (See Table 1, samples are marked with \*).

Specific metal(loid) for geochemical speciation analyses were chosen depending on their total amount in sediments to achieve reliable informations: As, Cr, Cu, Ni, Zn and Pb. Sequential selective extractions of metals and metalloids were carried out using a modified version of Tessier's protocol (Argese et al., 2003). Metal(loid)s were fractioned in 6 phases: exchangeable (phase I), bounded to carbonates (phase II), bounded to Fe/Mn oxide/hydroxides at different degree of crystallization (phase IIIA and IIIB), bounded to organic matter and sulphides (phase IV) and residual (phase V), actually, their definition has to be considered "operational" as it has to be referred to the specific extractive solution and physical-chemical condition used for metal(loid) mobilization. See supplementary Figure S1 for the detailed protocol. All analyses were performed in triplicate to assess the analytical reliability, ensuring a S.D. lower than 10%. The accuracy and reliability of the protocol has been verified by comparing the sum of metal(loid) concentrations in each fraction and the total metal(loid) content. This difference varied among 0-15%; samples exceeding this interval were not considered as acceptable.

#### **2.2.4. Metal(loid) determination in clams**

The total concentration of Arsenic (As), Cadmium (Cd), Chromium (Cr), Copper (Cu), Iron (Fe), Manganese (Mn), Nickel (Ni), Zinc (Zn), Lead (Pb) and Mercury (Hg) were measured in Manila clam samples. For metal(loid) quantification in clams, 6 individuals from each area were used: the whole soft tissues were freeze-dried, weighed and homogenized with liquid nitrogen.

After that, aliquots of about 0.15g of lyophilized soft tissue were microwave-digested in closed Teflon vessels in a CEM MDS-2000 microwave oven, with 4 ml of Milli-Q water, 4 ml of HNO<sub>3</sub> 65-67% and 4 ml of H<sub>2</sub>O<sub>2</sub> 30%. All analyses were performed in triplicate to assess the analytical reliability, ensuring a S.D. lower than 10%.

Quality controls were repeatedly performed by the means of reference materials (DORM-2 dogfish muscle – National Research Council Canada and NIST SRM 2976 mussel tissue – Community Bureau of Reference), which have been submitted to the same laboratory procedures as clam

samples. Metal(loid) determinations were performed by NexION 2000 ICP-MS (Perkin Elmer) and by Varian SpectrAA-250 Plus spectrometer equipped with a Varian VGA-77 vapour generation accessory (for Hg determination).

As already described by Velez et al. (2015b) the concentration of metal(loid)s in clams was expressed in mg per kg wet weight (w.w.), to compare with maximum permissible limits expressed in mg kg<sup>-1</sup> w.w., but also in dry weight (d.w.) according to Ponsero et al. (2009), to make comparisons with literature data and to allow the determination of the Biota-Sediment Accumulation Factor (BSAF).

The Biota-Sediment Accumulation Factor (BSAF) was determined dividing the total concentration of a given element in the soft tissue of an organism by the concentration of that element in the sediment (see Velez et al., 2014).

### **2.3. Multivariate Statistical analyses**

Data obtained were analyzed by multivariate analysis techniques, namely principal component analysis (PCA) and cluster analysis (CA), to investigate structures in data and to extract the relevant informations. PCA was firstly performed on the whole environmental variables concerning salinity, pH, Eh, granulometric distribution, TOM, total metal(loid) (As, Cd, Cr, Cu, Fe, Mn, Ni, Zn, Pb) content in the whole sediment and in each granulometric fraction (43 variables, data not shown). Hg was never included since it was always under the limit of detection.

Experimental data were then arranged in a single reduced matrix giving the best resolution yet comparable results in terms of explained variance.

The final matrix examined by PCA and CA was composed of 9 observations, i.e., sediment samples, and 16 variables, namely salinity, pH, Eh, sediment granulometric composition (granulometric fraction > 63 µm, 63- 25 µm, < 25 µm), TOM and total metal(loid) (As, Cd, Cr, Cu, Fe, Mn, Ni, Zn, Pb) content in the whole sediment. Geochemical speciation results were not included into this kind of statistics as they are incremental data type. Before performing either PCA

and CA analyses, data pre-treatment procedure were carried out, i.e., mean centering and unit variance scaling, to make order of magnitude and range of variation of all comparable variables. Regarding CA, the k-mean clustering algorithm and Ward's method were both used to group samples. All the statistical analyses were performed with the software PLS Toolbox 8.1 (Eigenvector Research, Inc., Wenatchee, WA, USA) for Matlab ©.

### 3. Results

#### 3.1. Sediments

##### 3.1.1. Physico-chemical characteristics

Sediment characteristics are fully described in Table 1.

Salinity ranged between 3.6 g/L (ÍL A) and 14.9 g/L (ÍL C), whilst temperature ranged from 19.5 (TR C) to 21.2 °C (TR A).

CN B and TR C showed peculiar characteristics for pH, Eh and total organic matter content (TOM) whose values were always different from the rest of the samples: pH ranged between 6.6 (CN C) and 7.39 (ÍL C), whereas CN B and TR C showed pH values of 7.75 and 7.80, respectively.

Eh ranged from -378 mV (ÍL C) to -45 mV (CN A) but were higher at CN B (-2 mV) and TR C (+51.8).

TOM values ranged from 3.96% (ÍL A) and 10.2% (TR B); CN B and TR C were the samples with the lowest TOM values (0.93% and 1.14%, respectively). Most of the nine samples presented a percentage of approximately 55-60% of sand (> 63µm), 30-40% of coarse silt (63-25 µm) and 5-8% of fine silt-clay (< 25 µm). On the opposite, CN B and TR C were characterized by the high percentage of the sand (> 63µm), representing the 100% and 98.7%, respectively.

##### 3.1.2. Total metal(loid) content in the whole sediments-and in the granulometric fractions

Whole sediment contamination is summarized in Table 2. It is noteworthy observing that Hg was always under the detection limits (L.O.D.) in all investigated sites. L.O.D. values for Hg for total digestion matrix corresponded to  $0.2 \times 10^{-3}$  mg/Kg (d.w.).

The remaining elements were generally present, showing a similar contamination pattern along the investigated sites, especially for the Ílhavo area. CN B and TR C presented a lower contamination for all elements, when compared to the other sites. Ni was under the detection limits (L.O.D.) in CN B sample. L.O.D. values for Ni for total digestion matrix corresponded to  $0.65 \times 10^{-3}$  mg/Kg (d.w.).



Elements exceeding threshold values for sediment toxicity are indicated in bold: As, Cd, Cr, Ni, Zn in all investigated areas, whilst Pb exceed the limits only at the ÍL area.

Data on contamination for distinct granulometric fractions are reported in Table 3.

L.O.D. values for Cd for total digestion matrix corresponded to  $0.05 \times 10^{-3}$  mg/Kg (d.w.).

Generally, samples characterized by low percentage of fines ( $<63\mu\text{m}$ ) also show low metal(loid) content, as is particularly evident for CN B and TR C. The fine fractions ( $63\text{--}25\mu\text{m}$  and  $<25\mu\text{m}$ ) hosted the higher concentrations of metal(loid)s in all investigated sites, despite their limited occurrence in the whole sediments. At CN B the sandy fraction ( $>63\mu\text{m}$ ) hosted the whole metal(loid) content, since it was the only granulometric fraction occurring in the whole sediment. TR C showed a metal(loid) concentration 50 times higher in the fine fraction compared to the sandy one, despite the fine fraction represented only the 1.2% of the whole sediment.

### 3.1.3. Geochemical speciation data

Results of geochemical speciation on the whole sediment are shown in Figure 2, expressed as the percentages of each extraction phase. Detailed data on element concentrations for each phase are reported in Table 4.

Details of L.O.D. limits for each digestion matrix (namely each phase) and each metal(loid) are reported in Breda (2017).

Each metal(loid) showed a peculiar behavior and a specific affinity with one or more phases.

Concerning As, except for CN B and TR C, all sites showed that almost 50% of As is in association with oxides/hydroxides of Fe and Mn (third phase A and B), thus potentially bioavailable under reducing conditions. The rest of As was in the residual phase, not a bioavailable form. CN B and TR C showed a percentage of 11% and 17% of As in the second phase, corresponding to a concentration of 0.17 mg/kg (d.w.) for both samples; 20% of As occurred in the third phase (A and B) and 69% (CN B)-62% (TR C) in the residual phase (Table 4).

CN B and TR C showed percentages of 87% and 82% of Cr in the residual phase (corresponding to a concentration of 4.3 mg/kg and 5.10 mg/kg, respectively). In the other sites, at least the 55% of Cr occurred in the residual phase; the rest was associated with oxides/hydroxides of Fe and Mn (third phase B) (Table 4).

Cu showed its particular affinity to organic matter and sulphides, and it was mostly associated with the fourth phase, ranging from 43.7 % (TR A) to 55.3% (ÍL C), which becomes bioavailable under strong oxidizing conditions. The rest of Cu was associated to the residual phase and a low percentage (1-5%) occurred in the second and third phases. Concerning CN B and TR C Cu was never detected in potentially bioavailable phases, occurring only in the residual one (3.97 mg/kg and 3.79 mg/kg respectively) (Table 4).

Ni showed specific affinity to the third phase, mostly bounded to Fe/Mn oxides/hydroxides (40-50%). A percentage between 14-17% was bounded to the fourth phase and 28-33% occurred in the residual one. A lower percentage (6-10%) was detected in the second phase, which becomes bioavailable under acidification. CN B and TR C showed that the majority of Ni occurred in the residual phase (71-62%) while the rest was bounded to the third phase A and B (17-28%) and to the second one (7-8%). A little fraction also occurred in the first phase (3-2%) corresponding to a concentration of just 0.04 mg/kg and 0.03 mg/kg, respectively (Table 4).

Zn had a unique behaviour, since it occurred in all the geochemical phases (Table 4). The first phase occurred in all the samples analyzed, varying from 3% (CN C) to 23% (TR C). Moreover, Zn showed high affinity to the third phase (A and B), and in fact it was mostly bounded to Fe/Mn oxides/hydroxides, ranging from 30% (CN C) up to the 50% (CN B).

Pb was mostly bounded to the second phase (34-44%), showing its affinity with carbonates with whom it (co-) precipitated. It was also bounded to the third phase A and B (30-35%) and to the fourth phase (4-13%). The rest was bounded to the residual phase (13-28%). CN B and TR C samples differentiated from others as almost all Pb (93%) occurred in the residual phase at concentrations of 8.57 mg/kg and 10.4 mg/kg, respectively (Table 4).

Third phase (A and B) were the preferential association phase for As, Cr, Ni, Zn and Pb, even if it also showed high affinity for the second phase. Cu was mostly associated to the fourth phase when organic matter occurred in the samples.

### 3.2. Metal(loid) content in clams, PTWI and BSAF

The results for the total element concentration in clams *R. philippinarum* are reported in Table 5, while BSAF values are shown in Table 6. Hg was never detectable in clam samples.

Elements concentrations in clams were slightly higher in the ÍL samples, particularly for As and Ni. Considering the maximum permissible limits defined by international organizations (EFSA, European Food Safe Authority; USFDA, United States Food and Drug Administration; FSANZ, Food Standards Australia and New Zealand; JECFA, Joint FAO/WHO Expert Committee on Food Additives) for the analyzed elements, As concentrations exceed the limits in all investigated areas considering the FSANZ limit.

The amount of clam soft tissues that a 70 kg adult needs to consume in one week to exceed the PTWI (Provisional Tolerable Weekly Intake) is shown in Table 5. The results indicated higher health risks concerning As in all investigated areas, since less than a quarter of Kg of clams (FW) consumed in one week is sufficient to exceed the PTWI.

However, FSANZ standard values, JECFA and FSANZ PTWI values are referred only to the inorganic As (iAs) content in seafood, whilst the results herein presented are referred to total As content in clams.

Moreover, BSAF values above 1 were observed for As within all the investigated areas, for Zn in CN and TR, and for Cd within TR area. For other elements, BSAF values were similar among the investigated areas and always lower than 1 (Table 6).

### 3.3. Multivariate analyses

The total variance accounted by the first two PCs was around 82%. Figures 3 and 4 show respectively the loading and score plots with respect to PC1 and PC2.

In Figure 3, PC1 explained 69% of the total variance, showing negative loadings for Eh, pH and sediment fraction  $>63\mu\text{m}$ , while positive loadings for all the other variables. PC2 explained the 13% of the residual variance, related mainly to the variables Eh and sediment fraction  $<25\mu\text{m}$  (positive loadings), pH, Cu, Pb and Zn (negative loadings). Finally, Cd and S (salinity) were not relevant in any of the two first PCs.

Score plot calculated for cases (Figure 4) showed that IL samples (A, B, C) grouped close to each other. Also CN A and CN C resulted very similar, while CN B and TR C were separated from the other samples.

The simultaneous analysis of the score and loading plots indicated that PC1 discriminates TR C and CN B from the other samples; in fact, they were the only samples characterized by negative scores on PC1, i.e. high pH, Eh,  $>63\mu\text{m}$  granulometric fraction, and low total metal(loid) content. PC2 allowed to separate the couple CN A and CN C, from the IL samples (A, B, C), as the former were characterized by the highest Eh, lowest pH and metal(loid) content.

The Cluster analyses performed with the two different clustering algorithm showed the same results. Moreover, CA results were consistent with those of PCA, hence dendrograms were not reported. In fact, CA identified two main branches, including three main groups. The first branch was composed by two separate clusters, the first one including CN A, CN C, TR A and TR B, and the second one the three IL samples (A, B, C). The cluster composed by the two sandy sites CN B and TR C formed the other tree branch.

The coherence of the results obtained with different statistical analyses proves the robustness of the results themselves.

## 4. Discussion

### 4.1. Sediment characteristics, contamination and geochemistry

The present study demonstrated that, overall, salinity, pH and temperature slightly changed among the sediments of the investigated areas, confirming the previously published studies (Velez et al., 2015a); a high variability for granulometric composition, Eh and TOM content was detected in Costa Nova, Torreira and Ílhavo areas. Differences were observed among, but also within studied areas.

CN B and TR C sites showed sandy granulometric composition, highest Eh values and very low TOM content. On the opposite, the other sites from Costa Nova (CN A, CN C), Torreira (TR A, TR B), and Ílhavo (ÍL A, ÍL B, ÍL C) showed a higher amount of silt-clay sediment fractions, negative or very negative Eh values and a higher TOM content.

Considering the whole sediment contamination, it is noteworthy observing that all metal(loid) concentrations found at CN B and TR C were from five to twelve times lower than those observed in the other sites. Mn concentrations at CN B and TR C were three times lower, Fe ten times lower, and Zn up to twenty times lower.

The remaining samples presented higher metal(loid) content, and especially for As, Cd, Cr, Ni above the limits established by the International standards (Field et al., 2002). ÍL samples showed also high Pb and Zn content, above the established limits. The results confirmed that, among studied areas, Ílhavo channel was the most contaminated area, as previously suggested in a recent study (Velez et al., 2015a). The results obtained in the present work also confirmed that Pb, Cr and As were the most abundant elements in the Ria de Aveiro Lagoon, while Cd and Hg were the least abundant ones (Velez et al., 2015a). Hg, in particular, was always under the threshold detection limit, in all the sediments herein analyzed.

Metal(loid) content data were also collected for the first time in the different granulometric fractions, showing that the fine fractions (63-25  $\mu\text{m}$  and  $< 25 \mu\text{m}$ ) hosted the highest metal(loid) content, compared to the sandy one ( $>63 \mu\text{m}$ ). This is particularly evident at TR C: despite the fine fraction represented only the 1.2% of the whole sediment, it showed metal(loid) concentrations up to fifty times higher than those observed in the sandy one. CN B represented the only exception, since it was characterized by a negligible fine fraction. Consequently, the metal(loid) content in CN B sediments represented the limited contamination level occurring in sandy sediments for Costa Nova area, in absence of fine fractions driving the majority of anthropogenic inorganic pollutants. The results obtained from single variables were also reinforced by multivariate statistical analyses, since both PCA and cluster analysis (CA) showed valuable correlations among the variables herein investigated. Significant correlations were observed among i) fine grained sediments, TOM and metal(loid) content and ii) sandy fraction, pH and Eh.

These results indicated that hydrodynamics played a major role in the Ria de Aveiro Lagoon, influencing both the grain-size distribution of particles and the contaminant fluxes (Lopes and Dias, 2007; Martins et al., 2015a). Tidal forces were distributed differently within the lagoon and they contributed to create depositional zones where fine-grained sediments, organic matter and contaminants could be preferentially accumulated, also due to high affinity among fine particles and metal(loid)s (Luoma, 1990).

On the opposite, areas where transport phenomena prevailed were characterized by higher percentage of sandy fraction, micro-oxidizing or oxidizing conditions, lower values of both TOM content and metal(loid) concentration, especially at the lagoon entrance and where higher tidal prism and current forces occurred. Hydrodynamics differentiated areas within the lagoon where intense currents affected bottom sediments from those areas where net deposition prevailed. High variability of granulometric composition, Eh, TOM and pH among and within investigated areas resulted from a complex and irregular hydrodynamic system, where different conditions coexisted close to each other.

Ílhavo was the example of a shallow and flat channel where geometry contributed to a significant dumping of current (Dias et al., 2000) and, consequently, to a scarce water and sediment exchange with other areas of the lagoon. Results herein obtained demonstrated that weak water exchange brought to the compartmentalization of this area and the consequent similarity among the Ílhavo samples (ÍL A, ÍL B, ÍL C) concerning Eh, grain size distribution and metal(loid) contamination. The scarce water exchange, the valuable occurrence of both fine grained-particles and organic matter led to the establishment of intense bacterial activity, driving anoxic conditions to the Ílhavo sediments.

S. Jacinto channel is considered one of the most important area of the lagoon, especially concerning hydrodynamics and sediment transport (Lopes et al., 2006), contributing for the 38% to the total tidal prism of the Ria de Aveiro Lagoon. Samples collected in S. Jacinto channel, namely at Torreira (TR A, TR B and TR C) showed the simultaneous occurrence of erosive and depositional sites. TR A and TR B were characterized by the presence of fine-grained sediments, negative Eh values and higher metal(loid) and TOM content, while TR C showed opposite features. PCA and CA results indicated that TR C differentiated from other samples of the same channel for all analyzed variables. In particular, its hydrodynamic conditions, together with the scarcity of fine grained particles, prevented the accumulation of TOM and metal(loid)s.

Mira channel contributed to the tidal prism of the lagoon for the 10%: despite its close position to the artificial lagoon inlet, the channel is separated from the inlet by a sand bar which reduces the entity and the speed of water fluxes (Silva, 1994). Samples collected there, namely at Costa Nova (CN A, CN B and CN C) showed different characteristics: CN A and CN C exhibited typical features of depositional areas, as the fine-grained particles content was remarkable. High TOM content and low redox condition are characteristics of anoxic environments, where microbial activity contributed to reduce the oxygen content. Also, metal(loid) accumulation was relevant for these two samples. CN B presented opposite characteristics, confirming the occurrence of very different biogeochemical patterns within the same areas of the Ria de Aveiro Lagoon.

These patterns influenced the geochemical speciation results, collected for the first time also from those areas subjected to edible bivalve exploitation. Previous geochemical data were collected for the Ria de Aveiro Lagoon (Martins et al., 2015a), but bioavailable fractions were identified with a different sequential extraction protocol and bioaccumulation patterns were investigated in benthic foraminifera (Martins et al., 2010; 2015b).

Considering the six phases obtained by the sequential extraction protocol herein applied, the readily bioavailable fraction is limited to the first one. For the analyzed sediments of the Ria de Aveiro Lagoon, the readily bioavailable fraction was detectable only for Cu, Ni and Zn; conversely, it was never detected for the other analyzed elements (As, Cr, Pb). The results herein obtained were especially compatible with the Zn properties, as it is generally distributed in all phases, with comparable values among them.

Potentially bioavailable fractions are the second, the third (A and B) and the fourth ones, and they could be solubilized only in particular conditions. Second phase is constituted by labile carbonates of biogenic or geochemical origin, deposited as part of the sediments in transitional environments. The results from the Ria de Aveiro Lagoon showed that the second phase was only relevant for Pb, probably occurring as co-precipitate of insoluble calcium carbonate. The carbonates are sensitive to pH variations, as they dissolve when pH decreases below certain limits ( $< 5$ ), determining the mobility of associated metal(loid)s (Du Laing, 2011). The pH values collected within the three investigated areas of the Ria de Aveiro Lagoon were not consistent with calcite solubilization, thus preventing the occurrence of bioavailable Pb forms. Elements associated to third phases A and B are those bounded to Fe/Mn oxide/hydroxides, and they can be mobilized consequently to a change of redox potential, depending on the crystallinity's degree of the oxide/hydroxides. The insoluble oxidized  $\text{Fe}^{3+}$  can be reduced to soluble  $\text{Fe}^{2+}$  and the insoluble oxidized  $\text{Mn}^{4+}$  can be reduced to soluble  $\text{Mn}^{2+}$  (Du Laing, 2011; Kalnejais et al., 2015). Thus, in reduced conditions, the solubilization of Fe/Mn oxide/hydroxides and of associated elements can occur (Aston and Chester, 1976). The results showed a considerable amount of As, Cr, Ni, Zn and Pb associated to the third



phase, and especially for Pb, this fraction was comparable to the fraction detected in the second phase. This scenario underlined the predominance of the third phase as the preferential phase of metal(loid) accumulation in the sediments of the Ria de Aveiro Lagoon, with the exception of Cu and Pb. The majority of elements are generally associated to the third phase, because Fe and Mn oxide/hydroxides are the preferred association forms for ions eventually occurring in the water column (Luoma and Rainbow 2008, Du Laing, 2011, Kalnejais et al., 2015).

Fourth phase is also known as the oxidable one, it is represented by elements bounded to organic matter and sulphides, which are ubiquitous in reduced transitional environments (Zonta et al., 1995). Results from this work showed the prevalence of the Cu in the fourth phase, reflecting the typical distribution pattern of this element. In fact, Cu has a great affinity for humic, fulvic acids and sulphides (Kerndorff and Schnitzer, 1980). Metal(loid)s associated to the fourth phase could become bioavailable in oxidizing conditions, subsequently to the oxidation of organic matter and sulphides, and then released in the water column.

Finally, the residual phase includes the element form which is never bioavailable, and generally considered the residual phase (Argese and Bettiol, 2001). Residual phase accounted for a varying percentage, depending on the specific metal(loid) and sediment composition: results herein collected showed that As, Cr and Cu presented a considerable residual phase. As generally occurs in the residual phase as Fe arsenate, characterized by high-grade crystallization and scarcely mineralizable.

#### **4.2 Manila clam contamination, BSAF values and risks for its consumption**

The bioaccumulation data collected from this study underlined that Manila clams showed As values exceeding the limits established by international organizations, namely FSANZ, in all investigated areas. FSANZ and JECFA limits were established for inorganic As (iAs), stating that this form should be preferentially analyzed for As risk assessment in seafood (JECFA, 2011). However, in this study we used total As content for risk assessment for two main reasons: 1) there is a lack of reliable iAs speciation protocols, especially concerning the rates for iAs calculation from total As

data (JECFA, 2011); 2) contrasting toxicity data regarding the different organic and inorganic As forms are emerging. Although the organic forms generally prevail in seafood, and they were considered not or less toxic than inorganic ones (JECFA, 2011), detailed toxicity studies on single As species are still ongoing (Luo et al., 2017 and citations within), confirming that also organic As forms – for example DMA (dimethyl Arsenic acid) can be toxic for human assimilation (Jansen et al., 2016). Moreover, the risk for As bioaccumulation in Manila clams and its transfer through the trophic chain was already proved by previous studies in Portuguese coastal ecosystems (Velez et al., 2015, b; Chiesa et al., 2018): this metalloid occurred at the highest concentrations in Manila clams from the Ria de Aveiro Lagoon, the Óbidos Lagoon and the Tagus Estuary. Previous data showed the ability of Manila clam to accumulate As above other elements (Velez et al., 2015a), posing risk for its consumption as a valuable seafood. Results from current work and from previous studies (Velez et al., 2015a, b; Chiesa et al., 2018) showed that less than 1 Kg of clams (FW) consumed in one week would be sufficient to exceed the PTWI for As in all the Portuguese coastal ecosystems. Also Ereira et al. (2015) raised concern about the possible adverse effects of consuming clam *Scrobicularia plana* from As contaminated areas in the Ria de Aveiro lagoon.

As a consequence, As represents the most dangerous element for health concern consuming clams from Portuguese coastal environments (Velez et al., 2016), and its occurrence in edible bivalves should be carefully monitored in the future (Ereira et al., 2015).

However, it has been already demonstrated that comparing element concentration in clams with sediment total contamination could fail to identify a direct relationship (Velez et al., 2015a,b; Chiesa et al., 2018). Previous studies conducted in the Ria de Aveiro Lagoon on Manila clam contamination (Velez et al., 2015a), showed that metal(loid) concentrations found in clams were not proportional to the metal(loid) occurrence found in the sediments. The same Authors stated that clams collected in areas of the lagoon with higher sediment contamination tend to present higher concentration of elements, but higher BSAF values were generally identified in clams from the less

contaminated areas. Within this study, the geochemical data were used for the first time to clarify the metal(loid) behaviour in the environment and the bioaccumulation patterns in Manila clams. Results herein collected showed that BSAF values  $> 1$  were identified only for As, Cd and Zn; the same results for As and Cd were observed in the Ria de Aveiro Lagoon from Velez et al. (2015a) (Zn was not analyzed in the cited paper).

As in the sediments was preferentially associated with the third phase (A and B), namely bounded to Fe/Mn oxides/hydroxides; these forms could solubilize in reducing conditions, promoting the mobilization of As and its transferring to the water column. In this form, As becomes bioavailable and could be easily bioaccumulated by biotic compounds. These results are in accordance with Martins et al. (2015a), who stated that not only anthropogenic activities, but also biogeochemical processes seem to be responsible for persistence, bioavailability and bioaccumulation of specific metal(loid)s in the Ria de Aveiro Lagoon.

BSAF values for Cd were  $> 1$  within TR area. Cd is an element generally characterized by high mobility, consequently bioaccumulated by biotic matrix. Cd concentrations in estuarine sediments are generally low, since it is easily mobilized in the estuarine environments to the water column, and bioaccumulated by benthic organisms. Moreover, the low Cd concentrations occurring in the sediments avoided the achievement of reliable geochemical speciation data.

BSAF values  $> 1$  for Zn could be justified considering that the geochemical speciation data showed the occurrence of a limited Zn fraction bounded to the first phase, those representing the directly bioavailable one, in all the areas. Thus, if the Zn was occurring in a bioavailable form, it could have been directly accumulated by the Manila clams living in the bottom sediments.

BSAF values were not relevant ( $< 1$ ) for all remaining metal(loid)s. Their destiny was different depending on their specific chemical features: e.g. in a reducing environment Cr (III) forms stable insoluble compounds, whilst Cu could be released while organic matter degrades and then precipitated as insoluble sulphides. These scenarios may explain the reason why not all metal(loid)s were bioaccumulated by clams, despite reducing condition can mobilize most of them. BSAF

values for Cr were very low (0.02-0.03), a coherent result with the predominant Cr form occurring in the third phase and in the residual one (not available).

Cu data also showed that geochemical speciation compared to BSAF values (0.18-0.30) could be a good indicator of the redox condition of the analyzed sediments in the Ria de Aveiro Lagoon. In fact, Cu solubilization after organic matter degradation did not occurred, due to the precipitation of newly formed sulphides.

Concerning BSAF values for Mn and Fe, they were low (0.14-0.17) and very low (0.02-0.03), since in the redox conditions of the analyzed sediments the colloid forms of Fe/Mn oxides/hydroxides could be solubilized in the interstitial water, releasing  $\text{Fe}^{2+}$  and  $\text{Mn}^{2+}$ , which precipitate with newly formed sulphides, together with other associated metal(loid)s. This process prevented the transferring of metal(loid)s to the water column.

BSAF values for Ni and Pb are in accordance with the scenario described above. Moreover, Pb was identified by geochemical speciation analysis also in the second phase. The low BSAF values observed for Pb suggested that the solubilization of carbonates did not occur, preventing the release and bioaccumulation of bioavailable Pb forms in the clams.

#### **4.3 Manila clam management strategies in the Ria de Aveiro Lagoon**

The sediment contamination, the geochemical speciation and the bioaccumulation patterns in biotic compounds are of particular concern for the environmental safety, conservation and sustainable exploitation of a multi-use protected area like the Ria de Aveiro Lagoon.

In the Ria de Aveiro, metal(loid) bioaccumulation has been investigated in many taxa above bivalves, such as foraminifera (Martins et al., 2010; 2015b), polychaetes (Freitas et al., 2012b; Pires et al., 2017), gastropods (Cardoso et al., 2013) and crustaceans (D'Ambrosio et al., 2013); among vertebrates, several fish species and their parasites have been studied (as examples Cid et al., 2001; Eira et al., 2009; Araujo et al., 2015). No data are currently available for bird species.

The system's natural capital is an important factor for the development of the municipalities situated in the Ria de Aveiro Lagoon area (Lopes et al., 2017), considering the complex ecosystem goods and services provided by the lagoon (Sousa et al., 2013; Lillebø et al., 2015).

According to Lillebø et al. (2015) multiple ecosystem services for the Ria de Aveiro Lagoon have been described- based on CICES (Common International Classification of Ecosystem Services)- regarding (i) provisioning services (as ex. wild plants and animals; fisheries and aquaculture; materials from plants and animals); (ii) regulation and maintenance (as ex. Bio-remediation and filtration, sequestration, storage of pollutants; mass stabilization and control of erosion; flood protection; maintenance of the nurseries; pest control), and (iii) cultural services (as examples educational, scientific and recreational activities).

Shellfish species, like Manila clam, can contribute to all these sections, as examples by fisheries and aquaculture (provisioning); by their role within bio-remediation and filtration/ sequestration/ storage of pollutants (regulation and maintenance), also representing valuable species for educational, scientific, recreational activities and typical local products (cultural).

The management of the Ria de Aveiro Lagoon has been largely improved in the last years to maintain these services (Lillebø et al., 2015): as an example, a new secondary treatment connected to the S. Jacinto outfall has effectively reduced the point source nutrient loads (ammonium and phosphate) into the lagoon, representing a step forward for the implementation of the WFD (Water Frame Directive), through eutrophication abatement (Lopes et al., 2017). However, additional efforts have to be made to ensure better coordination and conciliation between the governmental institutions and the different sectors of activities, as well as to safeguard the integration among a variety of planning and management tools (Lillebø et al., 2015).

Specifically concerning the metal(loid) contamination, it must be remembered that above the European Legislation referred to EFSA limits established for Cd, Pb, and Hg, no other National or Regional regulations exist for metal(loid) contamination in the Ria de Aveiro Lagoon. Moreover, EFSA regulations should be also implemented, since they do not provide specific limits for As

contamination, although As contamination of seafood is occurring. The local Regulation concerning Manila clam collection in the Ria de Aveiro Lagoon is limited to the minimum size for collection (40 mm) and to the collection method (only hand collection is permitted, during low tides). The National Portuguese legislation concerning the classification of areas exploited for bivalve collection has been recently revised and updated (IPMA, 2017), but specifically regarding microbiological pollution. In this context, four estuarine areas have been identified within the Ria de Aveiro Lagoon: three of them (including Mira channel) were classified as “B” (bivalves can be collected, but they must be destined to depuration, transposition or to industrial transformation), whilst Ílhavo channel has been classified as “C” (bivalves can be collected, but they must be destined to long –time transposition or to industrial transformation). None of the identified areas within the Ria de Aveiro Lagoon have been classified as “A”, namely safe for direct human consumption.

There is no specific regulation concerning the permitted/not permitted areas for clam collection based on metal(loid) contamination and/or bivalve bioaccumulation in the Ria de Aveiro Lagoon. As previously stated by Figueira and Freitas (2013) this aspect should be further considered in the Ria de Aveiro when evaluating an area for bivalve production, in order to guarantee that the use of available areas for shellfish collection can produce bivalves not only below standard maximum levels, but with the lowest element load possible, thus be more safe to consumers.

The results obtained from this study can reinforce the informations about the contamination patterns existing within the lagoon, the safety of bivalves as edible seafood and should contribute to new shellfish management plans within the Ria de Aveiro lagoon. As an example, the Ílhavo area, which emerged as the most sensitive one for metal(loid) bioavailability and their transferring through the trophic chain, should be considered in further plans. The monitoring of metal(loid) contamination in this area should be implemented, and clam collection should be eventually limited/prohibited, depending on the future changing scenarios.

## Conclusions

This study investigated the metal(loid) contamination of sediments and the bioaccumulation patterns of Manila clam from the Ria de Aveiro Lagoon, including for the first time geochemical speciation data. The holistic approach herein applied showed that multiple driving forces interacted together in a complex framework, determining site-specific impacts. These impacts may result in a local risk for the transferring of a determined metal(loid) from the abiotic to the biotic matrix and throughout the trophic chain. The data herein collected showed that the Ílhavo area is the most sensitive in terms of geochemical speciation of metal(loid) and bioaccumulation, thus it should be carefully monitored and controlled to avoid risks of clam consumption. Therefore, the data collected within this study may contribute to the understanding of metal(loid) dynamics in the Ria de Aveiro Lagoon, improving locally the management plans concerning clam collection and exploitation, eventually limiting or prohibiting the clam exploitation in specific areas. Moreover, the new approach herein proposed can be applied in other estuarine environments, especially those where metal(loid) contamination and commercial exploitation of benthic bivalves are occurring.

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**Table 1.** GPS coordinates of the three sampling areas and sediment physical-chemical characteristics of the nine sampling site: S = salinity (g/L); pH; T = temperature (°C); Eh = Redox potential (mV), TOM = total organic matter (%), grain size distribution of fraction (%) > 63µm; between 63 and 25 µm, <25 µm.

The \* symbol and bold lines indicate the sediment samples chosen for geochemical speciation.

Area	GPS	Site	S	pH	T	Eh	TOM	>63µm	63-25 µm	<25 µm
Costa Nova (CN)	N 40° 36' 28.3"/ W 008° 44' 46.0"	CN A	6.50	6.72	21.0	-45.0	7.41	55.0	38.0	8.00
		<b>CN B*</b>	<b>5.20</b>	<b>7.75</b>	<b>21.0</b>	<b>-2.00</b>	<b>0.93</b>	<b>100</b>	<b>0.00</b>	<b>0.00</b>
		<b>CN C*</b>	<b>12.0</b>	<b>6.60</b>	<b>21.0</b>	<b>-46.0</b>	<b>7.75</b>	<b>48.0</b>	<b>45.0</b>	<b>7.00</b>
Ílhavo (ÍL)	N 40° 36' 39.0"/ W 008° 40' 58.2"	ÍL A	3.60	7.28	20.7	-255	3.96	52.0	44.0	5.00
		ÍL B	12.3	7.20	20.6	-354	5.95	57.0	37.0	6.00
		<b>ÍL C*</b>	<b>14.9</b>	<b>7.39</b>	<b>20.7</b>	<b>-378</b>	<b>2.75</b>	<b>60.0</b>	<b>37.0</b>	<b>3.00</b>
Torreira (TR)	N 40° 45' 35.3"/ W 008° 41' 51.9"	<b>TR A*</b>	<b>12.2</b>	<b>7.12</b>	<b>21.2</b>	<b>-163</b>	<b>8.50</b>	<b>59.0</b>	<b>33.0</b>	<b>8.00</b>
		TR B	11.4	7.34	20.7	-333	10.2	58.0	38.0	4.00
		<b>TR C*</b>	<b>10.6</b>	<b>7.80</b>	<b>19.5</b>	<b>51.8</b>	<b>1.14</b>	<b>98.7</b>	<b>0.80</b>	<b>0.40</b>



**Table 2.** Whole sediment contamination (mg/kg d.w.). L.O.D. = Limit of detection.

L.O.D. values for Ni corresponded to  $0.65 \times 10^{-3}$  mg/Kg (d.w.), for Hg corresponded to  $0.2 \times 10^{-3}$  mg/Kg (d.w.). Threshold values for sediment toxicity are originally reported by Field et al. (2002).

Bold values represent values exceeding the threshold limits. N.A. = Not available data.

Site	As	Cd	Cr	Cu	Fe	Mn	Ni	Zn	Pb	Hg
CN A	<b>24.6</b>	<b>0.68</b>	<b>59.6</b>	16.5	33000	316	<b>24.4</b>	<b>96.1</b>	27.0	<L.O.D.
CN B	2.11	0.17	6.68	8.93	2627	119	<L.O.D.	8.65	9.59	<L.O.D.
CN C	<b>22.9</b>	<b>0.67</b>	<b>61.8</b>	17.4	34000	308	<b>27.3</b>	93.0	27.6	<L.O.D.
ÍL A	<b>24.2</b>	<b>0.44</b>	<b>60.2</b>	30.1	31200	274	<b>25.1</b>	<b>141</b>	<b>42.0</b>	<L.O.D.
ÍL B	<b>25.4</b>	<b>0.49</b>	<b>58.8</b>	30.6	32400	275	<b>24.7</b>	<b>139</b>	<b>43.5</b>	<L.O.D.
ÍL C	<b>25.5</b>	<b>0.56</b>	<b>58.1</b>	30.2	32300	276	<b>24.1</b>	<b>148</b>	<b>41.2</b>	<L.O.D.
TR A	<b>19.2</b>	0.32	<b>59.3</b>	18.9	24500	286	<b>29.2</b>	<b>112</b>	28.4	<L.O.D.
TR B	<b>18.9</b>	0.12	41.3	12.8	25500	294	<b>19.4</b>	84.2	17.8	<L.O.D.
TR C	4.84	<b>0.56</b>	1.33	3.73	2530	107	0.80	28.9	10.2	<L.O.D.
Threshold values	7.40	0.38	49	32	N.A	N.A.	15	94	30	0.14

**Table 3.** Total element content in each granulometric fraction, expressed in mg/kg (d.w.).

N.D.= not detectable; < L.O.D. = below the limit of detection. L.O.D. values for Cd corresponded to  $0.05 \times 10^{-3}$  mg/Kg (d.w.), for Ni corresponded to  $0.65 \times 10^{-3}$  mg/Kg (d.w.), for Hg corresponded to  $0.2 \times 10^{-3}$  mg/Kg (d.w.). Bold cells represent the proportion of each fraction in the whole sediment (expressed as %), reported in brackets, for comparison with contamination data.

Site	Fraction	As	Cd	Cr	Cu	Fe	Mn	Ni	Zn	Pb	Hg
CN A	>63 $\mu\text{m}$ (55%)	23.2	0.73	52.8	16.2	32500	314	23.9	96.6	26.5	<L.O.D.
	25-63 $\mu\text{m}$ (38%)	23.8	0.73	63.2	15.8	31900	303	21.4	90.4	26.3	<L.O.D.
	<25 $\mu\text{m}$ (8%)	35.9	<L.O.D.	82.0	19.7	37700	349	39.1	108	30.0	<L.O.D.
CN B	>63 $\mu\text{m}$ (100%)	2.11	0.17	6.68	8.93	2627	119	<L.O.D.	8.65	9.59	<L.O.D.
CN C	>63 $\mu\text{m}$ (48%)	26.1	0.70	63.3	19.7	37500	324	28.6	102	27.9	<L.O.D.
	25-63 $\mu\text{m}$ (45%)	20.4	0.75	58.5	14.9	31600	305	25.1	83.0	26.8	<L.O.D.
	<25 $\mu\text{m}$ (7%)	16.6	<L.O.D.	71.9	18.3	23600	223	32.7	99.0	30.2	<L.O.D.
ÍL A	>63 $\mu\text{m}$ (52%)	29.0	0.85	57.5	31.2	32900	270	24.9	148	42.6	<L.O.D.
	25-63 $\mu\text{m}$ (44%)	17.8	<L.O.D.	61.2	27.8	28400	264	24.5	129	39.4	<L.O.D.
	<25 $\mu\text{m}$ (4%)	26.5	<L.O.D.	67.3	32.3	33200	305	27.9	145	50.7	<L.O.D.
ÍL B	>63 $\mu\text{m}$ (57%)	29.2	0.86	57.0	31.2	34600	274	24.5	142	44.2	<L.O.D.
	25-63 $\mu\text{m}$ (37%)	19.6	<L.O.D.	60.3	29.4	29100	272	24.7	133	41.3	<L.O.D.
	<25 $\mu\text{m}$ (6%)	25.8	<L.O.D.	66.7	33.2	31700	296	27.7	147	50.2	<L.O.D.
ÍL C	>63 $\mu\text{m}$ (60%)	28.8	0.93	57.2	31.7	33800	276	24.2	153	41.9	<L.O.D.
	25-63 $\mu\text{m}$ (37%)	20.2	<L.O.D.	59.0	27.8	29800	274	23.8	141	39.5	<L.O.D.
	<25 $\mu\text{m}$ (3%)	24.8	<L.O.D.	64.7	30.8	33600	296	26.0	140	47.1	<L.O.D.
TR A	>63 $\mu\text{m}$ (59%)	18.2	0.55	54.0	18.1	20800	240	27.1	107	28.4	<L.O.D.
	25-63 $\mu\text{m}$ (33%)	20.7	<L.O.D.	66.4	20.0	31200	370	32.2	117	29.8	<L.O.D.
	<25 $\mu\text{m}$ (8%)	19.8	<L.O.D.	69.3	19.6	25000	279	32.5	130	23.1	<L.O.D.
TR-B	>63 $\mu\text{m}$ (58%)	23.7	0.21	47.7	15.4	19800	243	22.6	92.6	17.6	<L.O.D.

	<b>25-63 <math>\mu\text{m}</math> (38%)</b>	10.2	<L.O.D.	28.5	8.06	32600	357	13.2	68.8	16.0	<L.O.D.
	<b>&lt;25 <math>\mu\text{m}</math> (4%)</b>	32.1	<L.O.D.	70.3	19.7	41900	447	32.1	108	37.8	<L.O.D.
<b>TR C</b>	<b>&gt;63 <math>\mu\text{m}</math> (98.7%)</b>	4.76	0.57	0.74	3.59	2340	105	0.53	27.2	10.0	<L.O.D.
	<b>25-63 <math>\mu\text{m}</math> (0.8%)</b>	10.3	<L.O.D.	47.1	14.0	16100	266	19.6	159	26.9	<L.O.D.
	<b>&lt;25 <math>\mu\text{m}</math> (0.4%)</b>	15.0	<L.O.D.	55.6	19.7	23500	311	25.0	194	30.7	<L.O.D.

**Table 4.** Total element content in each geochemical speciation phase, expressed in mg/kg (d.w.).

< L.O.D. = below the limit of detection. Details of L.O.D. limits for each digestion matrix and each metal(loid) are reported in Breda (2017).

Site	phase I	phase II	phase III A	phase III B	phase IV	residual
<b>As</b>						
<b>CN B</b>	<L.O.D.	0.17	0.03	0.28	<L.O.D.	1.07
<b>CN C</b>	<L.O.D.	<L.O.D.	2.67	7.26	<L.O.D.	8.13
<b>IL C</b>	<L.O.D.	<L.O.D.	5.07	8.33	<L.O.D.	9.68
<b>TR A</b>	<L.O.D.	<L.O.D.	1.85	3.66	<L.O.D.	10.5
<b>TR C</b>	<L.O.D.	0.17	0.02	0.19	<L.O.D.	0.62
<b>Cr</b>						
<b>CN B</b>	<L.O.D.	0.07	0.17	0.31	0.09	4.3
<b>CN C</b>	<L.O.D.	0.39	1.01	16.7	0.14	26.1
<b>IL C</b>	<L.O.D.	0.42	1.45	17.9	0.24	24.3
<b>TR A</b>	<L.O.D.	0.16	0.96	16.1	0.29	24.3
<b>TR C</b>	<L.O.D.	0.12	0.19	0.73	0.11	5.10
<b>Cu</b>						
<b>CN B</b>	<L.O.D.	<L.O.D.	<L.O.D.	<L.O.D.	<L.O.D.	3.97
<b>CN C</b>	0.09	0.17	0.23	0.34	5.88	5.45
<b>IL C</b>	0.23	0.92	0.26	0.43	12.4	8.21
<b>TR A</b>	0.07	0.13	0.16	0.47	5.95	6.83
<b>TR C</b>	<L.O.D.	<L.O.D.	<L.O.D.	<L.O.D.	<L.O.D.	3.79
<b>Ni</b>						
<b>CN B</b>	0.04	0.09	0.02	0.21	0.03	0.95
<b>CN C</b>	<L.O.D.	1.31	1.68	9.07	3.07	5.99
<b>IL C</b>	<L.O.D.	1.73	1.51	5.81	2.97	5.86
<b>TR A</b>	<L.O.D.	2.08	2.12	8.43	3.01	6.06

<b>TR C</b>	0.03	0.13	0.04	0.41	<L.O.D.	1.01
<b>Zn</b>						
<b>CN B</b>	2.02	2.58	4.67	3.46	3.01	2.4
<b>CN C</b>	4.09	13.2	12.5	33.8	19.1	37.9
<b>IL C</b>	7.43	15.1	22.6	33.9	20.1	38.44
<b>TR A</b>	4.63	19.6	16.1	39.5	16.5	31.6
<b>TR C</b>	4.98	1.97	6.36	4.51	2.98	1.3
<b>Pb</b>						
<b>CN B</b>	<L.O.D.	0.32	0.1	0.24	<L.O.D.	8.57
<b>CN C</b>	0.00	5.28	1.24	3.81	0.69	4.35
<b>IL C</b>	0.00	12.2	3.32	5.33	3.59	3.59
<b>TR A</b>	0.00	6.66	1.03	5.20	0.88	4.50
<b>TR C</b>	<L.O.D.	0.39	0.02	0.38	<L.O.D.	10.4

**Table 5.** Clam contamination in three investigated areas, expressed in mg/kg w.w. Hg was not detectable in all clam samples. When available, Maximum permissible limits (ML) defined by international organizations are provided: EFSA, European Food Safe Authority; USFDA, United States Food and Drug Administration; FSANZ, Food Standards Australia and New Zealand; JECFA, Joint FAO/WHO Expert Committee on Food Additives. Values in bold are those exceeding the limits. PTWI (provisional tolerable week intake) and amount of clams that a 70 kg adult needs to consume to exceed PTWI for each element are also provided. Values in bold represent the areas where a higher risk occurs for clam consumption (less than 1 Kg for week). Table modified from Velez et al. (2015a, b).

Area	As	Cd	Cr	Cu	Fe	Mn	Ni	Zn	Pb
<b>Clams contamination (mg kg<sup>-1</sup> w.w.)</b>									
<b>CN</b>	<b>4.80</b>	0.18	0.23	0.61	95.4	6.18	0.03	14.3	0.80
<b>ÍL</b>	<b>6.84</b>	0.16	0.29	0.66	99.0	4.79	0.05	15.6	0.78
<b>TR</b>	<b>5.82</b>	0.16	0.25	0.61	93.7	4.79	0.04	14.0	0.90
<b>ML (mg kg<sup>-1</sup> w.w.)</b>									
<b>EFSA</b>		1.00							1.50
<b>USFDA</b>	86.0	4.00	13.0				80.0		1.70
<b>FSANZ</b>	1.00	2.00							2.00
<b>PTWI (mg kg<sup>-1</sup> w.w.)</b>									
<b>JECFA</b>	0.015	0.007		3.50			0.035		0.025
<b>FSANZ</b>	0.015	0.007							0.025
<b>Amount of clams consumed per week to exceed PTWI (Kg) based on JECFA values</b>									
<b>CN</b>	<b>0.22</b>	2.72		402			81.7		2.19
<b>ÍL</b>	<b>0.15</b>	3.06		371			49.0		2.24
<b>TR</b>	<b>0.18</b>	3.06		402			61.2		1.94

**Table 6.** BSAF values in three investigated areas for each analyzed element. The bold and \* symbol represent BSAF values exceeding 1. Hg was not detectable in all clam samples.

Area	As	Cd	Cr	Cu	Fe	Mn	Ni	Zn	Pb
CN	<b>1.68*</b>	0.37	0.02	0.30	0.02	0.17	0.26	<b>1.26*</b>	0.07
ÍL	<b>2.28*</b>	0.86	0.02	0.18	0.03	0.15	0.26	0.91	0.06
TR	<b>2.54*</b>	<b>1.09*</b>	0.03	0.32	0.03	0.14	0.31	<b>1.19*</b>	0.09

**Figure Captions**

**Figure 1. Sampling locations.** Sampling areas in the Ria de Aveiro Lagoon.

**Figure 2. Geochemical speciation of the whole sediments.** Results of the geochemical speciation of the whole sediments from the analyzed sites in the Ria de Aveiro Lagoon. Costa Nova (CN B, CN C); Ílhavo (ÍL C); Torreira (TR A, TR C).

**Figure 3. PCA loading plot of variables distribution.** Loading plot on the first two PCs of the 16 variables: pH; S = salinity (g/L); Eh = Redox potential (mV), TOM = total organic matter (%); grain size distribution of fraction (expressed as %) <25  $\mu\text{m}$ , between 25 and 63  $\mu\text{m}$ , > 63 $\mu\text{m}$ ; and total metal(loid) content (As, Cd, Cr, Cu, Fe, Mn, Ni, Zn, Pb). Hg was not included since it was always under the limit of detection. As an example, Cd = Cd total content in sediment samples (expressed as mg/Kg d.w.).

**Figure 4. PCA loading plot with sampling sites distribution.** Score plot on PC1 and PC2 of the 9 sampling sites from three different areas: Costa Nova (CN A, CN B, CN C); Ílhavo (ÍL A, ÍL B, ÍL C); Torreira (TR A, TR B, TR C).

**Supplementary Figure S1. Geochemical Speciation protocol.** Sequential extraction protocol for geochemical speciation with different phases.



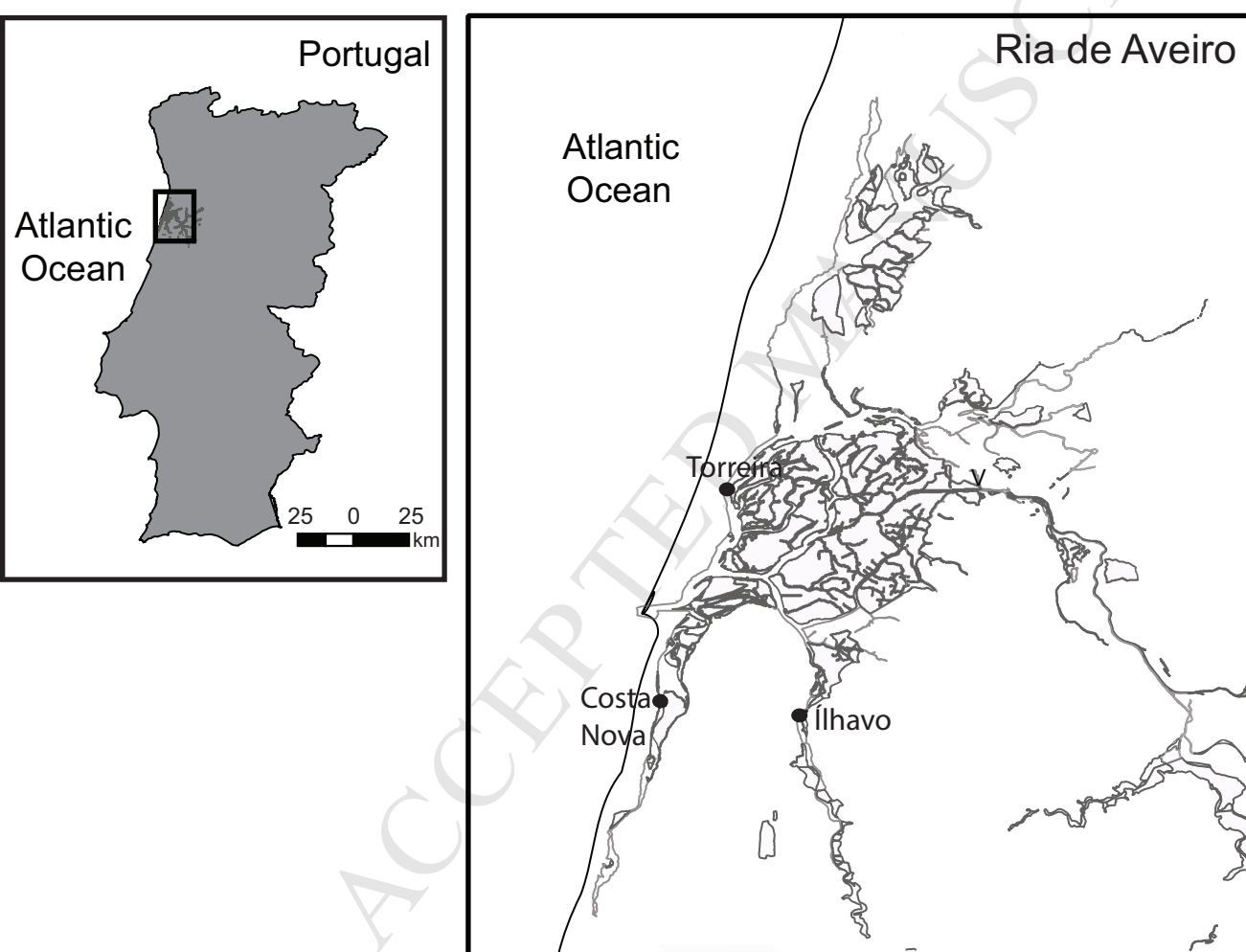
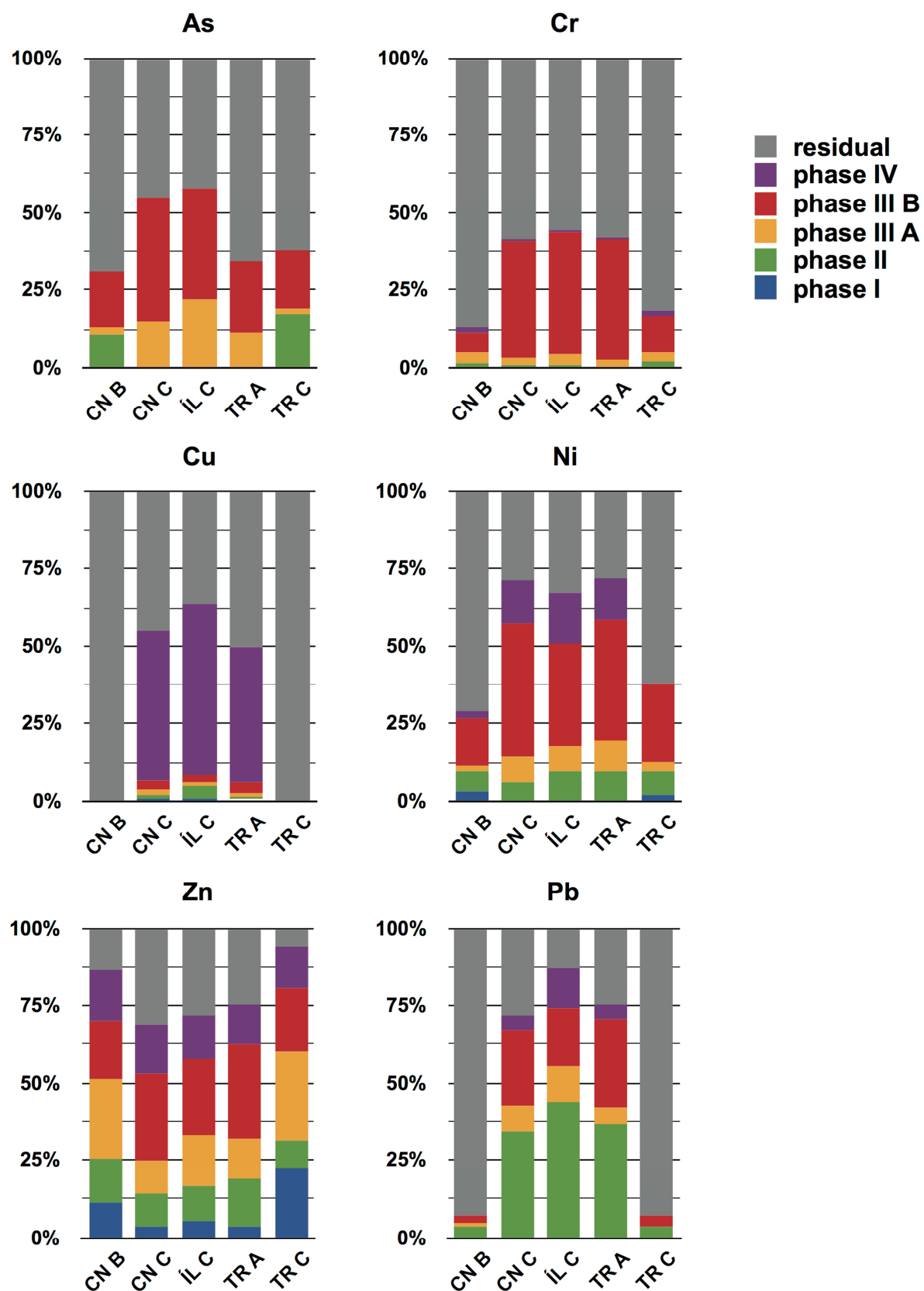
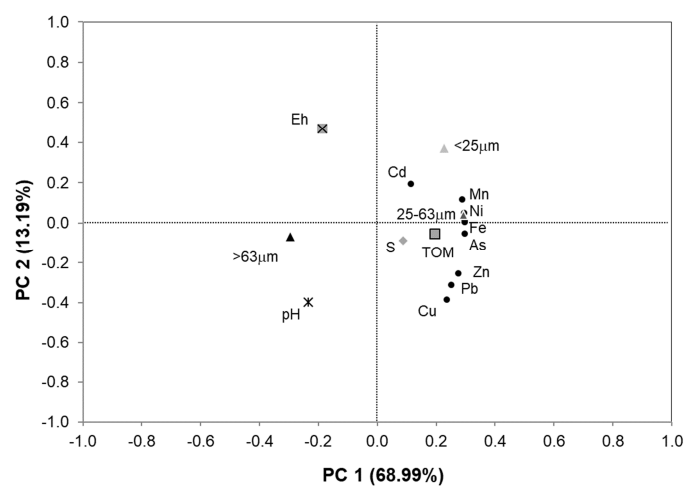
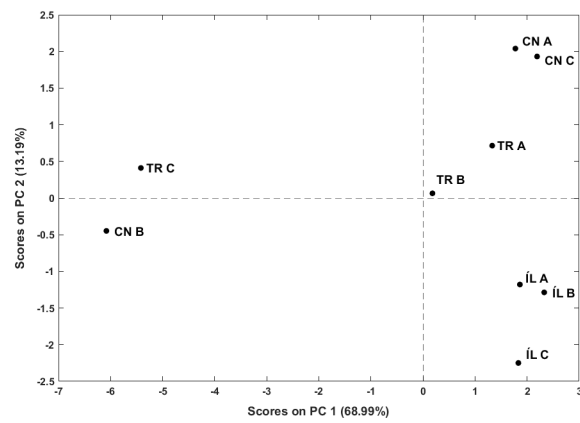


Figure 1







- metal(loid) fate has been investigated in the Ria de Aveiro Lagoon (Portugal)
- bioaccumulation patterns have been studied together with geochemical speciation data
- multiple driving forces interacted together to determine site- specific impacts
- bioaccumulation processes in Manila clam were observed for Cd, Zn and especially As
- the Ílhavo area should be considered the most dangerous in terms of clam consumption